MycoScience
Specialists in Microbiology & Regulatory Affairs

PDI EFFICACY STUDY OF SINGLE USE IMPREGNATED TOWELETTES FOR USE AS A SANITIZER FOR FOOD CONTACT SURFACES

Mycoscience, Inc. Study Protocol GLP-15-015, Rev. 00

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PURPOSE OF THE STUDY

To determine the sanitizing activity of a pre-saturated wipe on contaminated smooth glass and rough plastic (textured cutting board) 2' x 2' (4ft.^{2}) surfaces.

TEST SYSTEM AND JUSTIFICATION

Pre-saturated towelettes for sanitizing food contact surfaces will be tested via a modification of the methodology established for sanitizer towlettes in the US Environmental Protection Agency document EPA/AD Method Guidance #02: **Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes,** to meet the efficacy data requirements in the **OCSPP 810.2300: Sanitizers for Use on Hard Surfaces – Efficacy Data Recommendations**, EPA 712-C-07-091, September 4, 2012, **(h) Towelette Sanitizers for Food Contact Surfaces**

The test microorganism to be used in this study will be ***Shigella boydii*** **ATCC # 9207**.

TEST SUBSTANCES

1) Backspin No-Rinse FCSS, (8" x 10" Towel Size), Lot #7912-AE-937-047B, Active: 360 ppm BTC 1210 quat; DOM: 4/22/15; Exp. Date: 4/22/16

TEST SUBSTANCE CHARACTERIZATION

The identity, strength, purity, stability, solubility, and chemical composition of the test material are the responsibility of the sponsor.

CONTROL SUBSTANCE

Towelettes without active ingredients (e.g. AOAC Neutralizing Blank Solution only)

METHODS

The study will be a modification of the EPA/AD Method Guidance #02: **Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes,** and the Official Methods of Analysis of AOAC International, 17th Edition, 2013, Section 6.3.03, AOAC Official Method 960.09, **Germicidal and Detergent Sanitizing Action of Disinfectants**

PROCEDURE

1.0 The surfaces used in this study will consist of **smooth glass and rough plastic (textured cutting board) 2' x 2' (4ft.^{2}) surfaces**. One lot of the test substance will be tested in triplicate against the test microorganism inoculated and dried onto 2' x 2' glass and rough plastic surfaces.

1.1 The ***Shigella boydii*** culture will be prepared as in AOAC 960.09 C, with the final suspension preparation made by harvesting the culture from the surface of Nutrient Agar B, and will be standardized using a spectrophotometer. In order to support claims as a "one-step" cleaner – sanitizer, an organic soil load (5% fetal bovine serum) will be incorporated in the inoculum. The surfaces will be inoculated such that a minimum of 7.5×10^7 CFU of the test culture is recovered from a 2' x 2' surface after drying. A 2' x 2' surface will be comprised of eight 6" x 12" glass or rough plastic surface sections. Each 6" x 12" surface section will be inoculated with 0.125mL of the prepared inoculum suspension for a total inoculum of 1.0mL per 2' x 2' total surface area. The inoculum will be spread uniformly over each surface section using a sterile spreading stick. Due to the size of the surfaces being tested and in order to maintain sterility, all inoculated surfaces will be dried at room temperature and humidity for 25 minutes in a biological safety cabinet prior to testing. The dried surfaces will be used for testing immediately after preparation.

1.2 One wipe will be used to wipe an entire 2' x 2' inoculated surface. The wipe will be folded in half two times prior to wiping the first 6" x 12" surface section. Each surface will be wiped in a consistent manner up and down the surface and working from left to right across the entire surface, then back from right to left. This will be repeated one additional time so that the entire inoculated 6" x 12" surface section has been wiped in this manner two times. A total wipe time of 20 seconds per 6" x 12" surface carrier will be utilized. The wipe surface will be rotated and re-folded as necessary for each surface section wiped, so that the maximum of wipe surface is used over the course of wiping an entire 2' x 2' surface. After wiping, each surface section will be allowed to sit for 30 seconds and then will be transferred to a sterile composite bag containing 3,000mL of AOAC neutralizing blank solution. 30 seconds after wiping the last surface section, the wipe will be transferred to a sterile jar containing 200mL of AOAC neutralizing blank solution. The composite bag containing the surface carriers, and the jar containing the wipe, will be sonicated for 5 minutes, followed by thorough agitation by hand.

1.3 Surface and wipe extract suspensions will be assayed for surviving numbers of microorganisms using membrane filtration technique. Appropriate aliquots, such as but not limited to 3mL and 30mL of the sample surface extract, and 2mL and 20mL of the wipe extracts, will be filtered through individual sterile bacterial retentive filters followed by a 50mL rinse with AOAC neutralizing blank solution. The membrane filters will be transferred to the surface of Tryptone Glucose Extract Agar (TGEA-N) plates containing AOAC stock neutralizers, and will be incubated for 48 hours at 35 - 37°C. After the incubation period the plates will be enumerated.

Note: Due to the size of the test carriers, materials required, the time required to perform the associated testing, etc., a typical test day consists of running one lot of test substance on one test surface (glass or textured) in triplicate, and running one positive (parallel) control surface and wipe.

1.4 A parallel control count will be performed using a wipe with the active ingredient(s) omitted for the inoculated type of surface. The wipe will be saturated with AOAC neutralizing blank solution. The inoculated surface will be wiped, and the total 2' x 2' surface and wipe will be assayed for numbers of microorganisms as in 1.2 – 1.3 above. Representative dilutions of the surface and wipe extracts will be filtered, rinsed, plated, incubated, and enumerated as above. This will serve as the "zero-time" bacterial numbers recovery.

1.5 All reagents/neutralization media used will be as in AOAC 960.09 A or will otherwise be noted. Note: Neutralization media or neutralizer concentrations may be modified as necessary to provide the adequate level of effectiveness required by the neutralizer validation procedure. A sterility check will be performed on sterile media and materials used in the study.

1.6 To demonstrate the absence of residual antimicrobial effect in the neutralizer medium, 100 – 1,000 CFU of the test microorganism will be inoculated to a jar containing a test wipe and 200mL of AOAC neutralizing blank solution. A control wipe without active in AOAC neutralizing blank solution will be run for comparison. The jars will be sonicated, filtered (20mL aliquots), plated, and incubated as in section 1.2 – 1.3 above. Comparable growth on these plates after incubation will confirm neutralizer effectiveness.

Surviving organisms will be confirmed as *S.boydii* by microscopic (Gram Stain) and macroscopic examination.

Calculations

Microorganism percent reduction counts are calculated by the following formula:

$$\frac{\text{CFU Recovered (Sample Surface \& Wipe)}}{\text{CFU Recovered (Parallel Control Surface \& Wipe)}} \times 100 = \% \text{ Surviving CFU}$$

100 - % Surviving CFU = % Reduction

STATISTICAL METHODS

N/A

RESULTS

To be considered valid, results must meet standard effectiveness: at least a ≥99.999% mean reduction in the numbers of test microorganisms on the treated surface over that of the parallel control surface in 30 seconds. Results will be reported according to actual count and percent reduction over the parallel control count.

REPORT

The final report will include an identification of all test articles, summary of the methods used, any modifications to the study, results, summary, and any other pertinent information.

QUALITY ASSURANCE

The Quality Assurance Department will conduct periodic inspections at adequate intervals to ensure the integrity of this study. The Quality Assurance Department will prepare and submit a signed statement to the sponsor with the final report. This GLP study will be conducted according to the Good Laboratory Practice Regulations set forth in 40 CFR: Part 160.

RECORDS

All documentation, data, and final reports derived from this study will be retained in the archives at Mycoscience, Inc., 25 Village Hill Rd., Willington, CT, 06279.

REFERENCES

1) EPA Product Performance Test Guidelines, OCSPP 810.2300: Sanitizers for Use on Hard Surfaces – Efficacy Data Recommendations, EPA 712-C-07-091, September 4, 2012, (h) Towelette Sanitizers for Food Contact Surfaces (See Attachment A.)
2) Draft Interim EPA/AD Method Guidance #02, Dated April 12, 2001: Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes (See Attachment B.)
3) Official Methods of Analysis of AOAC International, 17th Edition, 2013, Section 6.3.03, AOAC Official Method 960.09, Germicidal and Detergent Sanitizing Action of Disinfectants (See Attachment C.)

PROPOSED DATES

Proposed Experimental Start Date: 4/28/15
Proposed Experimental Termination Date: 5/1/15

APPROVALS

Study Director: _____ Date: 4/27/15
Mycoscience, Inc.

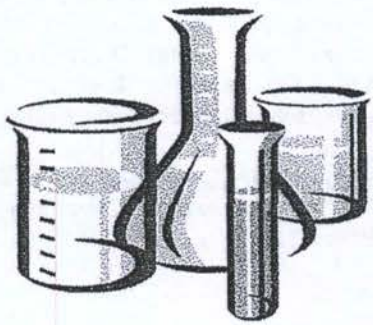
Sponsor Approval: _____ Date: 4/24/15
PDI, Inc.

United States Environmental Protection Agency
Office of Chemical Safety and Pollution Prevention (7510P)
EPA 712-C-07-091 September 4, 2012

EPA

Product Performance Test Guidelines

OCSPP 810.2300: Sanitizers for Use on Hard Surfaces—Efficacy Data Recommendations



(h) Towelette Sanitizers for Food Contact Surfaces. This section addresses efficacy testing for products with a label recommendation for the treatment of hard, non-porous surfaces which may come into contact with food. Food contact surface (FCS) towelettes are intended to be used to sanitize the following surfaces: hard non-porous tables, countertops (stainless steel, laminated, sealed ceramic) stove tops, interior and exterior surfaces of microwaves and refrigerators. FCS towelettes may not be used to sanitize the following food contact surfaces: utensils, glasses, food containers, dishes, unfinished wood cutting boards and cutting blocks, drain boards and food processing equipment. This list is not meant to be all-inclusive, but to serve as general guidance for the appropriate use of this type of antimicrobial pesticide. The Agency reserves the right to accept or deny use sites for food contact surface towelettes on a case-by-case basis.

(1) Test Procedure. The Agency recommends the use of the Interim Guidance for Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes. This guidance may be found at: http://www.epa.gov/oppad001/towelette.htm

Three samples, representing three different batches, one of which is at least 60 days old, should be evaluated for efficacy against *E. coli* (ATCC 11229) and *S. aureus* (ATCC 6538). Based on the claims, a variety of surfaces may be treated with the product. Each of the different types of test surfaces claimed may be used in the efficacy testing of the product (i.e., glass, stainless steel, plastic, and ceramic). At a minimum, the applicant should test: 1) a stainless steel or glass surface, and 2) a plastic with a rough surface (i.e., plastic cutting boards). At a minimum, the surface should equal four square feet. Inoculate the test surface with the challenge microorganisms. After inoculation, the test surface should be dried for 40 minutes in an incubator at 30 - 37°C. A "zero-time" bacterial numbers recovery test should be performed to demonstrate the efficiency of the recovery process, and should be reported.

The towelette removed from its container should be handled with sterile gloves. The inoculated surfaces should be tested by wiping the surfaces with the saturated towelette. One towelette should be used to wipe the number of carriers that equal four square feet. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping the contaminated test surface.

(2) Evaluation of towelette sanitizing success. The product should demonstrate a ≥99.999% mean reduction in the number of test microorganisms (bacteria) within 30 seconds. The result should be reported according to the actual count and percentage reduction over the control.

Draft Interim Guidance for Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes

April 12, 2001

SUBJECT: Draft Interim Guidance for Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes

The attached draft, interim guidance has been developed for use by AD staff to provide guidance to applicants regarding appropriate methodology that should be utilized when conducting efficacy testing for pre-saturated towelettes. It is a model protocol, which if followed, is likely to provide the type of testing required by the Agency.

This document addresses the Agency's recommendations for evaluating the non-residual sanitizing efficacy of antimicrobial products (specifically pre-saturated towelettes) after application to hard, inanimate surfaces with which food may come in contact.

Specifically, the guidance provides details on the following:

1. purpose and scope of the guidance document,
2. test substance,
3. test methods,
4. reporting of data,
5. test standard, (which includes discussion of the test organisms, procedure, organic soil load, single pack towelettes versus roll of towelettes, towelette size and treatment surface area, and data generation),and
6. performance standard.

This draft, interim guidance should be followed when evaluating efficacy protocols for products of this type. This document may be released to the public when requested. If you have any questions, please contact your branch chief, team leader, or Laura Morris-Bailey.

Attachment

EPA/AD/Method Guidance #02 April 12, 2001

Draft Interim Guidance for AD Staff

Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelette

1. Purpose and Scope

The purpose of this document is to provide interim guidance for the evaluation of the sanitizing efficacy of antimicrobial products, specifically pre-saturated towelettes, after application to hard inanimate, nonporous surfaces. This approach may be used to substantiate bacterial sanitizing claims for pre-saturated towelettes applied on hard inanimate surfaces in food contact areas (such as counter tops in restaurants, kitchen cabinets, etc.). This guidance is limited to single use towelettes in both commercial and residential environments. Note: This guidance does not address products for use on utensils, glasses, food containers, dishes, and food processing equipment.

2. Test Substance

Unless otherwise specified, antimicrobial pesticides are to be tested with the formulation to be offered for sale using the product packaged in the same packaging intended to be marketed. Towelettes are a unique combination of antimicrobial chemical products pre-packaged as a unit in fixed proportions for application. Therefore, the complete products, as packaged in the manner to be offered for sale, must be tested according to the directions for use to insure efficacy as a hard surface sanitizer. The product tested must be from three batches as referenced in Section 5.6. Simulated re-use is not required since the product is intended to be removed from the package, used immediately, and discarded after use.

3. Test Methods

Test antimicrobial products in accordance with the proposed directions for use. Depending upon the type of antimicrobial agent, target microorganisms, and the site to be treated, all tests are to address those factors that would normally be expected to be encountered in the use pattern intended for the product, including, but not limited to, the method of application; the nature of the surface (i.e., hard non-porous surface), item surface to be treated; the presence or absence of soil or other interfering conditions; ambient temperature and exposure period of 30 seconds.

Modification of the standard AOAC Germicidal Spray Products Test, official final method, (Official Methods of Analysis of the AOAC International. Chapter 6, Disinfectants, Official Method 961.02 Germicidal Spray Products as Disinfectants, Seventeenth edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417) is appropriate for this scenario. Instead of spraying the inoculated surface of the glass slide (as noted in the AOAC Germicidal Spray Products Test method), the towelette product is tested by wiping the surface of the glass slide with the saturated towelette, and then subculturing the slides after a 30 second exposure time. Liquid expressed from the used towelette needs to be subcultured separately. Subcultures of the liquid expressed from the used towelettes are expected to be negative for growth.

4. Reporting of Data

Systematic and complete descriptions of the tests employed and (see item #3 above) the results obtained are essential for proper review and evaluation of product performance by the Agency. All test reports must include identification of the testing laboratory or organization, when and where the tests were conducted and the name of the person(s) responsible for conducting the tests and those who prepared the study report.

5. Test Standard

The following parameters need to be taken into account when developing efficacy data for sanitizing activity of towelettes used on hard inanimate surfaces:

1. Test Organisms

Testing is to be based upon an adequately controlled in-use study or simulated in-use study. At a minimum, the microorganisms that must be tested are Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 11229). Testing also must be undertaken for any additional microorganisms that are claimed on the label. The starting inocula of the test microorganisms must be of sufficient concentration to provide between 75 - 125 x 106 cfu/ml on the parallel control surface.

2. Procedure

Based on the claims, a variety of surfaces may be treated with the product. Each of the different types of test surfaces claimed may be used in the efficacy testing of the product (i.e., glass, stainless steel, plastic, and ceramic). At a minimum, the applicant must test:1) a stainless steel or glass surface, and 2) a plastic with a rough surface (i.e., plastic cutting boards). Inoculate the test surface with the challenge microorganisms. After inoculation, the test surface is dried for 40 minutes in an incubator at 30 - 37EC. A "zero-time" bacterial numbers recovery test must be performed to demonstrate the efficiency of the recovery process, and must be reported.

The towelette is removed from its container and handled with sterile gloves. The inoculated surfaces are to be tested by wiping the surfaces with the saturated towelette. The area of the towelette used for wiping is rotated so as to expose a maximum amount of its surface in the course of wiping the contaminated test surface. After wiping the contaminated surface with the towelette, all remaining liquid is to be expressed from the used towelette into an empty sterile container and subcultured separately. Run parallel tests on the towelette (as well as expressed liquid from the used towelette) with the active ingredients omitted in an identical manner to serve as the control.

After the 30 second contact time, recover the test microorganisms by washing the treated surfaces with adequate agitation in an appropriate media or dilution fluid containing appropriate neutralizers. Enumerate microorganisms on appropriate nutrient agar, containing the same neutralizers, by the pour or spread plate technique.

The environmental conditions, such as relative humidity and temperature, employed in the test must also be reported. These conditions must be the same as those likely to be encountered under normal conditions of use.

3. Organic Soil Load

For products making one-step sanitization claims, the test surface must have an organic soil load applied to the surface prior to the initial treatment and challenge (at a minimum 5% bovine serum). The organic soil level indicated is considered appropriate for simulating lightly or moderately soiled surface conditions. When the surface to be treated has heavy soil deposits, a cleaning step must be required on the label prior to the application of the antimicrobial agent. In the absence of testing with an organic soil load, a one-step claim cannot be made and a pre-cleaning step is required and must be noted on the label.

4. Single Pack Towelettes Versus Roll of Towelettes

There may be more moisture retained in a towelette from single pack towelettes than in a towelette from a roll of towelettes. If the towelette roll container does not remain closed, there is a possibility that the towelettes at the end of the roll may not contain as much moisture as those towelettes at the start of the roll. Therefore, to ensure continued efficacy, the label needs to state that the towelette must be visibly wet (saturated) before use, and that the surface treated must be visibly wet after use.

5. Towelette Size and Surface Area

At this time, there are no limitations/restrictions regarding the size of the towelette. The Agency's suggested minimum surface area to be treated per towelette is 2' x 2'. However, the size of the surface area treated must be representative of the area that the towelette will treat effectively and reflective of the surface area to be tested in the study. The size of the surface area to be treated, as demonstrated by the data, must also be stated on the label as the recommended maximum surface area to be treated.

6. Data Generation

Three samples, representing three different batches, one of which is at least 60 days old, must be evaluated for efficacy against Escherichia coli (ATCC 11229) and Staphylococcus aureus (ATCC 6538). Testing for additional microorganisms claimed on the label is to be conducted on two batches of product. Tests are to be conducted in triplicate.

6. Performance standard

The product must demonstrate at least a 99.999% reduction in the number of test microorganisms (bacteria) within 30 seconds. The result must be reported according to the actual count and percentage reduction over the control.

Guidance approved as Agency standard April 12, 2001 by the Office of Pesticide Programs/Antimicrobials Division.

6.3.03

AOAC Official Method 960.09
Germicidal and Detergent Sanitizing Action of Disinfectants

First Action 1960
Final Action
Revised First Action 2011
Revised First Action 2013

(Suitable for determining minimum concentration of chemical that can be permitted for use in sanitizing precleaned, nonporous food contact surfaces. Minimum recommended starting concentration is 2–4× this concentration. Test also determines maximum water hardness for claimed concentrations. These microbiological methods are technique-sensitive, in which careful adherence to the method with identified critical control points, good microbiological techniques, and quality control is required for proficiency and validity of results.)

Notes: (*1*) All manipulations of the test organism are required to be performed in accordance with appropriate biosafety practices stipulated in the institutional biosafety regulations. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms, refer to the CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* manual.

(*2*) Sanitizers may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Personal protective clothing or devices are recommended during the handling of these items for purposes of activation, or efficacy testing. A chemical fume hood or other containment equipment may be employed when appropriate during performance of tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for specific product/active ingredient to determine best course of action.

(*3*) References to water (H_2O) mean reagent grade, except where otherwise specified.

(*4*) Commercial media (e.g., TSA) made to conform to the specified recipes may be substituted.

(*5*) Alternate organism preparation procedures may be used for test organisms not mentioned herein.

A. Reagents

(a) *Culture media.*—(*1*) *Nutrient agar A (NA-A).*—Boil 3.0 g beef extract, 5.0 g peptone [BD Biosciences (1 Becton Dr, Franklin Lakes, NJ 07417, USA), Codified Cat. No. 211840, or equivalent; special grades must not be used], and 15 g salt-free agar in 1 L H_2O. Do not use premixed, dehydrated media. Dispense 10 mL portions in 20 × 150 mm tubes or 20 mL portions in 25 × 150 mm tubes and sterilize. Slant to cool. Use for daily transfer of test culture.

(*2*) *Nutrient agar B (NA-B).*—Boil 3.0 g beef extract, 5.0 g peptone (BD Biosciences, Codified Cat. No. 211840, or equivalent; special grades must not be used), and 30 g salt-free agar in 1 L H_2O. Steam sterilize for 20 min at 121°C. Dispense approximately 25 mL portions into sterile 20 × 100 mm Petri plates. Used for the generation of final test culture.

(*3*) *Nutrient agar (NA).*—Dissolve Bacto agar to 1.5% in nutrient broth and adjust to pH 7.2–7.4. Steam sterilize for 20 min at 121°C. Dispense into sterile Petri plates. Used for the generation of frozen stock cultures for *E. coli*.

(*4*) *Nutrient broth (NB).*—Boil 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (i.e., anatone) in 1 L H_2O for 20 min and dilute to volume with deionized water; adjust to pH 6.8 ± 0.1. Filter through paper (Whatman No. 4, or equivalent), place 10 mL portions in 20 × 150 mm test tubes, and steam sterilize 20 min at 121°C. Used in the preparation of nutrient agar plates.

(*5*) *Tryptic Soy Agar (TSA).*—Prepare according to manufacturer's instructions. Used in the generation of frozen stock cultures for *S. aureus*.

(*6*) *Tryptic Soy Broth (TSB).*—Prepare according to manufacturer's instructions. Used in the generation of frozen stock cultures for *S. aureus*.

(b) *Subculture media.*—Choose the appropriate recovery agar and neutralizer to inactivate the test substance. Suggestions include:

(*1*) *Tryptone glucose extract agar with neutralizer (TGEA-N).*—BD Biosciences, Codified Cat. No. 223000. Combine 24 g dehydrated media with 975 mL H_2O and 25 mL stock neutralizer if necessary, and steam sterilize at 121°C.

(*2*) *Tryptone glucose extract agar (TGEA).*—BD Biosciences, Codified Cat. No. 223000.

(c) *Neutralizer stock solution.*—Mix 40 g Lecithin (granular), 280 mL polysorbate 80, and 1.25 mL 0.25 M phosphate buffer stock solution (PBSS); dilute with H_2O to 1 L and adjust to pH 7.2. Dispense in 100 mL portions and sterilize.

(d) *Neutralizer blanks.*—Mix 100 mL neutralizer stock solution, 25 mL 0.25 M PBSS, and 1675 mL H_2O. Dispense 9 mL portions into 20 × 150 mm tubes and sterilize. Alternate neutralizers may be used as necessary.

(e) *Phosphate buffer stock solution (PBSS).*—0.25 M. Dissolve 34 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1 M NaOH, and dilute to 1 L.

(f) *Phosphate buffer dilution water (PBDW).*—Add 1.25 mL 0.25 M PBSS to 1 L H_2O. Dispense in 99 mL portions and sterilize.

Table 960.09A. Percent light transmission at various wavelengths corresponding to approximate bacterial concentrations

% Light transmission with filters, nm							Average bacterial CFU/mL
370	420	490	530	550	580	650	
7.0	4.0	6.0	6.0	6.0	7.0	8.0	13.0×10^9
8.0	5.0	7.0	7.0	7.0	8.0	9.0	11.5
9.0	6.0	8.0	8.0	8.0	9.0	10.0	10.2
10.0	7.0	9.0	9.0	9.0	11.0	11.0	8.6
11.0	8.0	10.0	10.0	10.0	12.0	13.0	7.7
13.0	9.0	12.0	12.0	12.0	13.0	15.0	6.7

Table 960.09B. Preparation of $BaSO_4$ suspensions corresponding to approximate bacterial concentrations

Standard No.	2% $BaCl_2$ solution, mL	1% H_2SO_4 (v/v) solution, mL	Average bacterial CFU/mL
1	4.0	96.0	5.0×10^9
2	5.0	95.0	7.5
3	6.0	94.0	8.5
4	7.0	93.0	10.0
5	8.0	92.0	12.0
6	10.0	90.0	13.5
7	12.0	88.0	15.0

(g) *Cryoprotectant solution (TSB with 15% glycerol).*—Suspend 7.5 g TSB in 212.5 mL deionized water. Add 37.5 g glycerol and stir until dissolved; boil to dissolve completely. Dispense into bottles and autoclave for 15 min at 121°C.

(h) *Tween-80 (polysorbate 80).*

(i) *PBS (1×) with 0.1% (v/v) Tween 80 (PBS + Tween 80).*—Add 100 mL PBS 10× solution and 1 mL Tween 80 to a volumetric flask; fill with deionized water to the 1000 mL mark and mix thoroughly. Sterilize by filtration.

(j) *Sterile water.*—Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. *See Standard Methods for the Examination of Water and Wastewater.*

(k) *Test organisms.*—Use *Escherichia coli* ATCC No. 11229 or *Staphylococcus aureus* ATCC 6538. Prepare frozen stock cultures and use for up to 18 months.

(l) *Preparation of frozen stock cultures.*—Open one ampule of lyophilized organism as indicated by ATCC (or other reputable vendor). Using a tube containing 5–6 mL TSB for *S. aureus* or NB for *E. coli*, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix well. Incubate broth culture at 36 ± 1°C for 24 ± 2 h. Using a sterile spreader, inoculate a sufficient number of TSA plates for *S. aureus* or NA plates for *E. coli* (e.g., 5 to 10 plates per organism) with 100 µL each of the culture. Incubate plates at 36 ± 1°C for 24 ± 2 h. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% glycerol) to the surface of each plate. Resuspend the cells using a sterile spreader and aspirate the cell suspension from the surface of the agar. Repeat by adding another 5 mL cryoprotectant to the agar plates, resuspend the cells, aspirate suspension, and pool with the initial cell suspension. Alternatively, 10 mL cryoprotectant solution may be added per plate for resuspending with subsequent aspiration. Transfer the suspension into a sterile vessel. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, dispense approximately 1.0 mL aliquots into cryovials (e.g., 1.5 mL cyrovials). Place and store the cryovials at –70°C or below; these are the frozen stock cultures. Store frozen stock cultures for up to 18 months. Reinitiate cultures using a new lyophilized culture vial. These cultures are single-use only.

B. Apparatus

(a) *Glassware.*—250–300 mL wide-mouth Erlenmeyers; 100 mL graduate; Mohr, serological, and/or bacteriological (APHA specification) pipets; 20 × 150 mm and 25 × 150 mm test tubes. Sterilize at 180°C in hot air oven ≥2 h or steam sterilize for a minimum of 20 min at 121°C with a drying cycle.

(b) *Petri dishes.*—Sterile, 20 × 100 mm.

(c) *Whatman No. 2 filter paper.*—Sterile.

(d) *Water bath.*—Controlled at 25 ± 1°C.

(e) *Transfer loops.*—Make 4 mm id single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA, USA). Fit other end in suitable holder. Bend loop at 30° angle with stem. Commercially available 4 mm id transfer loops may also be used. Volumetric transfer devices may be used instead of transfer loops (e.g., micro volume pipet).

(f) *Spectrophotometer.*—Calibrated.

(g) *Timer.*—For managing timed activities, any certified timer that can display time in seconds.

C. Preparation of Test Culture

Defrost a single cryovial of frozen stock culture at room temperature and briefly vortex to mix. Streak one loopful of the thawed frozen stock onto an NA-A slant and incubate at 36 ± 1°C for 24 ± 2 h. Only one daily transfer is required prior to the initiation of the final test culture. For the final test culture, add 5 mL PBDW to an NA-A slant (daily culture). Using a sterile loop, dislodge growth from agar surface. Collect mixture and transfer to a flask containing 99 mL PBDW. Mix thoroughly. Use 200 µL of the mixture to inoculate a minimum of 5 NA-B plates and to create a bacterial lawn. Incubate at 36 ± 1°C for 24 ± 2 h. After incubation, add a minimum of 5 mL PBS + Tween 80 to each plate. Using a sterile rod, gently dislodge culture from agar surface; avoid disrupting agar. Combine culture from all plates and mix thoroughly. Filter collected culture through sterile Whatman No. 2 filter paper using a vacuum source; collect filtered culture into a sterile vessel. Standardize the test culture, if necessary using PBDW, to achieve a final test culture microbe population between 1.0×10^8 CFU/mL and 1.0×10^{10} CFU/mL (9–10 logs/mL).

D. Preparation of EDTA Solution and Standardization

(a) *EDTA standard solution.*—Dissolve 4.0 g $Na_2H_2EDTA{\cdot}2H_2O$ and 0.10 g $MgCl_2{\cdot}6H_2O$ in 800 mL H_2O and adjust by subsequent dilution so that 1 mL of solution is equivalent to 1 mg $CaCO_3$ when titrated. A total of 200 mL in addition to the original 800 mL H_2O should yield the proper concentration of 1000 ppm. Check EDTA solution after preparation or, if commercially purchased, against $CaCO_3$ standard at least every 2 months. Final concentration of solution should be 1000 ppm ± 5%. Used as the titrating solution for the Buret titrating method I.

(b) *Calcium carbonate standard solution in water.*—1 mL = 1 mg $CaCO_3$. Weigh 1.0 g $CaCO_3$, dried overnight or longer at 105°C, into 500 mL Erlenmeyer and add dilute HCl (1:2 aqueous solution) through funnel until $CaCO_3$ is dissolved. Add 200 mL H_2O, boil to expel CO_2, and cool. Add few drops methyl red indicator (0.1% in ethanol) and adjust color to intermediate orange with dilute NH_4OH or HCl as required. Transfer quantitatively to 1 L volumetric flask and dilute to volume. *Note:* Alternatively, calcium carbonate standard solution in water can be prepared using a commercially available NIST traceable concentrated standard (i.e., 10 000 ppm $CaCO_3$

ampule). Add one 10 mL standard ampule of 10 000 ppm as $CaCO_3$ to a 100 mL volumetric flask. Add deionized water up to volume mark. Mix thoroughly and transfer to a bottle. Use within 24 h of preparation. Used for the standardization of EDTA standard solution.

(c) *Buffer solution.*—67.5 g NH_4Cl and 570 mL NH_4OH diluted to 1 L with H_2O or acceptable alternative volume (e.g., 100 mL total volume).

(d) *Inhibitor solution.*—5.0 g $Na_2S{\cdot}9H_2O$ or 3.7 g $Na_2S{\cdot}5H_2O$ dissolved in 100 mL H_2O.

(e) *Indicator solution.*—0.5 g Chrome Black T in 100 mL 60–80% alcohol.

(f) *Standardization of EDTA through titration.*—Dilute 5–25 mL calcium carbonate standard solution in water (depending on hardness) to 50 mL with H_2O in Erlenmeyer (e.g., 10 mL calcium carbonate standard solution in water into 40 mL H_2O). Add 1 mL buffer solution, 1 mL inhibitor solution, and two drops indicator solution; mix thoroughly. Titrate with EDTA standard solution (using a calibrated Buret) slowly, by stirring continuously, until last reddish tinge disappears from solution, adding last few drops at 3–5 s intervals.

Note: As an alternative method for the *Standardization of EDTA*: In a beaker add 10 mL calcium carbonate standard solution in water, 40 mL deionized water.

(g) *Calculations.*—

Hardness as mg $CaCO_3$/L =
(mL EDTA standard solution × 1000)/mL test sample
($CaCO_3$ standard solution)

E. Preparation of Synthetic Hard Water and Titration

Method I

(a) *Solution 1.*—Dissolve 31.74 g $MgCl_2$ (or equivalent of hydrates) and 73.99 g $CaCl_2$ in boiled distilled water and dilute to 1 L or acceptable alternative volume (e.g., 250 mL total volume). Solution 1 may be heat or filter sterilized.

(b) *Solution 2.*—Dissolve 56.03 g $NaHCO_3$ in boiled distilled water and dilute to 1 L or acceptable alternative volume by adjusting ratio of $NaHCO_3$ to distilled water (e.g., 250 mL total volume). Solution 2 must be filter sterilized.

(c) *Hard water preparation.*—Solutions 1 and 2 are mixed thoroughly to prepare water at different hardness. Add Solution 1 (use 1 mL for each 100 ppm of hardness/L) to a volumetric flask. Each mL Solution 1 will give a water equivalent to ca 100 ppm of hardness calculated as $CaCO_3$ by formula:

Total hardness as ppm (µg/mL) $CaCO_3$ =
2.495 × ppm (µg/mL) Ca + 4.115 × ppm (µg/mL) Mg

[pH of all test waters ≤2000 ppm (µg/mL) hardness should be 7.6–8.0. Check prepared synthetic waters chemically for hardness at time of tests, using following method or other methods described in APHA, *Standard Methods for the Examination of Water and Wastewater*, 21st Ed., 2005.]

Prepare the following solutions volumetrically. Add approximately ¾ of total water volume to be prepared to a volumetric flask containing Solution 1. Add Solution 2 (4 mL/L) to the volumetric flask. Dilute with deionized water to volume mark. *For example:* 400 ppm hard water sample = add 4 mL Solution 1 and 4 mL Solution 2. Dilute to 1 L with deionized water.

(d) *Titration of hard water sample.*—Add 20 mL of hard water sample to 30 mL of deionized H_2O in an Erlenmeyer flask. Add 1 mL buffer solution, 1 mL inhibitor solution, and two drops indicator solution; mix thoroughly. Titrate hard water sample with EDTA standard solution (using a calibrated Buret) slowly, by stirring continuously, until last reddish tinge disappears from solution, adding last few drops at 3–5 s intervals. A 1 L hard water sample should yield 400 ppm water hardness. Adjust volumes of Solutions 1 and 2 depending on the required water hardness. Alternatively, the hard water sample can be titrated using a digital titrator, as in Method II.

(e) *Calculations.*—

Water hardness as mg $CaCO_3$/L = (mL of EDTA standard solution × 1000)/mL hard water sample with water

Method II
(Prepare solutions volumetrically)

(a) *Hard water (calcium carbonate solution).*—Prepare hard water using a commercially available standard. A NIST traceable standard is recommended (i.e., 10 000 ppm $CaCO_3$ standard ampules). To prepare a 100 ppm as $CaCO_3$ hard water sample, add one 10 mL standard ampule of 10 000 ppm as $CaCO_3$ to a 1000 mL volumetric flask. Add 990 mL deionized water (up to the volume mark). Mix thoroughly and transfer to a bottle. Titrate sample to verify water hardness as in Method II. Use within 24 h of preparation.

To prepare hard water samples of different hardness, adjust the standard volume accordingly. For example, to prepare a hard water sample of 400 ppm as $CaCO_3$, add four (10 mL) standard ampules of 10 000 ppm to a 1000 mL volumetric flask. Add 960 mL deionized water (up to the volume mark). Mix thoroughly and transfer to a bottle. Titrate sample for water hardness verification as in Method II. Use within 24 h of preparation.

Note: The pH of all test water ≤2000 ppm (µg/mL) hardness should be 7.6–8.0. Check prepared synthetic waters chemically for hardness at time of tests, using the following method or other methods described in APHA, *Standard Methods for the Examination of Water and Wastewater*, 21st Ed., 2005.

(b) *Hard water titration with digital titrator.*—Verify hard water hardness by titrating the sample to determine total water hardness. Titration can be conducted using a commercially available digital titrator. Digital titrators are available in kit form and include required titrating solutions, buffers, and color indicators. Use a digital titrator with the capacity to determine water hardness from $CaCO_3$ based hard water samples. Water hardness should be within the range of ~10 to 5% of the target hardness. For example, a target water sample of 400 ppm could have a hardness value range of 360 to 420 ppm and still be deemed valid.

(c) *Calculations.*—Report water hardness as mg $CaCO_3$/L. Follow digital titrator instructions for hard water hardness calculations.

F. Sanitizer Sample Preparation

Equilibrate water bath and allow it to come to 25 ± 1°C or the temperature specified (±1°C). Prepare the disinfectant dilutions within 3 h of performing the assay. Ready-to-use products are tested as received; no dilution is required. Aseptically prepare sanitizer samples. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use ≥1.0 mL or 1.0 g of sample sanitizer to prepare the use-dilution to be tested. Use v/v dilutions

for liquid products and w/v dilutions for solids. Rounding to two decimal places toward a stronger product is favorable. Dispense 99 mL aliquots of the diluted sanitizer or ready-to-use product into sterile wide mouth Erlenmeyer flask. Place flask in the equilibrated water bath for approximately 10 min to allow test chemical to come to specified temperature.

G. Neutralization Confirmation Test

A neutralization confirmation test must be performed in advance or concurrently with the efficacy test. Historical use of neutralizers for specific active ingredients may also be taken in consideration.

(*1*) *Test culture titer (TCT).*—Add 0.1 mL of the test organism serially diluted to target between 10–100 CFU/mL to 10 mL PBDW and mix thoroughly. Dilutions 10^{-4} and 10^{-5} should provide the range of 10–100 CFU/mL. Hold the mixture for a minimum of 2 min. Plate 0.1 mL aliquots in duplicate onto TGEA. Incubate plates at 36 ± 1°C for 24–30 h and record number of colonies.

(*2*) *Neutralization confirmation treatment (NCT).*—Add 1 mL of the test substance to 9 mL of the prescribed neutralizer and mix thoroughly. Within 30 s, inoculate the sample with 0.1 mL of the test organism used for the TCT. Mix thoroughly. Hold the mixture for a minimum of 2 min. Plate 0.1 mL aliquots in duplicate onto TGEA-N. Incubate plates at 36 ± 1°C for 24–30 h and record number of colonies.

(*3*) *Neutralizer toxicity treatment (NTT).*—Add 0.1 mL of the test organism used for the TCT to 10 mL of the prescribed neutralizer and mix thoroughly. Hold the mixture for a minimum of 2 min. Plate 0.1 mL aliquots in duplicate onto TGEA-N. Incubate plates at 36 ± 1°C for 24–30 h and record number of colonies.

(*4*) *Neutralization results and calculations.*—In order to demonstrate effective neutralization of the sanitizer, differences between treatments should not exceed 1.0 log (e.g., TCT minus NCT).

H. Operating Technique

Measure 99 mL of the germicidal solution at the concentration to be tested into sterile, 250 mL wide-mouth Erlenmeyer flask and place in constant temperature bath until it reaches 25 ± 1°C. Prepare one flask per test microbe, for each germicide to be tested. Also prepare similar flask containing 99 mL sterile PBDW to be tested for numbers control.

Add 1 mL of the test culture to each test flask as follows: Whirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at point of contact with germicidal solution. Add suspension midway between center and the inner edge of the flask with tip of pipet slightly immersed in germicidal solution. Avoid touching pipet to neck or side of flask during addition. Swirl flask to thoroughly mix contents. At 30 s after addition of the culture, transfer a 1 mL portion to a tube containing 9 mL neutralizer and mix well. This corresponds to 10^{-1} dilution tube.

For numbers control, add 1 mL of the test culture to 99 mL sterile PBDW in same manner as the treated sample. Within 30 s of addition of test culture, transfer 1 mL aliquot from the test flask (test culture × PBDW) into 9 mL neutralizer and mix well. This corresponds to 10^{-1} dilution tube.

Numbers control plating.—Make serial 10-fold dilutions in 9 mL PBDW, out to 10^{-6}.

Plate four 1 mL aliquots and four 0.1 mL aliquots from the 10^{-6} dilution to individual sterile Petri plates. This will result in 10^{-6} and 10^{-7} dilutions, respectively. Pour media, A(b)(*2*), cool to solidify, and invert. Incubate plates at 36 ± 1°C for 24–30 h. Alternatively, spread plating may be used instead of pour plating.

Treated sample plating.—From 10^{-1} tube (i.e., 9 mL neutralizer tube inoculated with 1 mL of exposed culture), plate four 1 mL aliquots and four 0.1 mL aliquots to individual sterile Petri plates. Pour media, A(b)(*1*), cool to solidify, and invert. Incubate plates at 36 ± 1°C for 24–30 h. Alternatively, spread plating may be used instead of pour plating.

I. Results

For numbers control.—For the test to be considered valid, the numbers control must fall between 7.0–8.0 logs.

For treated sample.—In order for the sanitizer to be deemed effective, a 5 log reduction in count of the number of microbes within 30 s is necessary.

J. Sterility Controls

(a) *Neutralizer.*—Plate 1 mL from previously unopened tube of neutralizer onto TGEA.

(b) *Sterile water.*—Plate 1 mL from each type of water used onto TGEA.

(c) *PBDW.*—Plate 1 mL from previously unopened tube of PBDW onto TGEA.

(d) *Germicide.*—Plate 1 mL of the germicide onto TGEA.

Additional Guidance

The information provided in this section is not considered a component of the official test; rather it serves as procedural guidance to augment germicidal and detergent sanitizing action of disinfectants testing of specific antimicrobial products and specific test conditions as the need arises.

A. Calculations

(*1*) *To calculate CFU/mL, use the following equation:*

$$\text{CFU/mL} = \frac{(\text{avg CFU for } 10^{-a}) + (\text{avg CFU for } 10^{-b})}{10^{-a} \text{ and } 10^{-b}}$$

where 10^{-a} and 10^{-b} are the dilutions plated. Four plates per dilution are plated for treated samples and numbers control samples. Use counts of 0 to 300 for calculation purposes. Score counts >300 as TNTC (too numerous to count).

(*2*) Calculate the mean $\log_{10}$ density for numbers control plates.

(*3*) Calculate the mean $\log_{10}$ density for treated sample plates.

(*4*) Calculate the $\log_{10}$ reduction (LR) for treated sample:

$$\text{Log}_{10} \text{ reduction} = \text{mean } \log_{10} \text{ numbers control} - \text{mean } \log_{10} \text{ treated sample}$$

Retesting guidance.—For tests where the product meets the performance standard and the numbers control mean $\log_{10}$ density value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standard and the numbers control mean $\log_{10}$ density is below 7.0, no retesting is necessary.

References: *Am. J. Public Health* **38**, 1405(1948).
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JAOAC **41**, 541(1958); **56**, 308(1973).
Official Methods of Analysis (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Methods 955.14, 955.15, 964.02.
ASTM International Method E 1054–Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents.

Standard Methods for the Examination of Water and Wastewater (2005) 21st Ed., American Public Health Association, Washington, DC, USA.

Posted: November 28, 2011; February 2013

Form B: Nice-Pak & PDI Analytical Request for Regular Test		Submission #: AN-15-B-43	Date: 4/23/15
		Project#: 7912	Submitted By: Anu Eapen
Purpose of Analysis:	Test BTC 1210 in the expressed liquid by Manual titration for Backspin No-Rinse FCSS Project		Department: PDI Surface
Formula Name /Reference#:	Backspin No-Rinse FCSS 7912-AE-937-046B	Notebook Reference#:	Backspin No-Rinse FCSS 7912-AE-937-047B
Applicator Name/Code:	100% Polyester-3OP540	Applicator Composition:	100% Polyester
Towel Width /Weight/Count:	8" X 10"	Packaging Components:	NP 3895
Check List For Stability Test: NA			
Requested Test and Actives Information: Analyze BTC 1210 in the expressed liquid by Manual titration for Backspin No-Rinse FCSS wipes			
Test Method: TM# Manual Titration, TM 200 and 208.		Sample Return ☒ or Disposal ☐	

Requested Tests	RT
SAMPLE ID	7912-AE-937-047B
BTC 1210 (%)	0.0360
pH	10.92
Odor*	Alcohol
Color*	Colorless
Appearance*	Clear
References	7912-AE-937-047B
Initials/Test Dates	AE, 4/23/15

Analyst Initials:	Start Date:	Completion Date:
AAE	4/23/15	4/23/15